

AN INTEGRIN HETERODIMER AND AN ALPHA SUBUNIT THEREOFFIELD OF THE INVENTION

The present invention relates to a recombinant or isolated integrin heterodimer comprising a subunit $\alpha 11$ and a subunit β , the subunit $\alpha 11$ thereof, homologues and
5 fragments of said integrin and of said subunit $\alpha 11$, processes of producing the same, polynucleotides and oligonucleotides encoding the same, vectors and cells comprising the same, binding entities binding specifically to binding sites of the same, and the use of
10 the same.

BACKGROUND OF THE INVENTION

Integrins are heterodimers composed of non-covalently associated α - and β -chains which connect cells to the extracellular matrix or to other cells (1). In addition
15 to acting as mechanical links between the cytoskeleton and extracellular ligands, integrins are signal transducing receptors which influence processes such as cell proliferation, cell migration and cell differentiation (2-4). Integrins can be grouped into subfamilies based on
20 shared β -chains, shared ligand binding properties, or shared structural features of the α -chains. Currently 17 α -chains and 8 β -chains have been identified (5). Of the subfamilies with shared β -chains, the $\beta 1$ subfamily has the most members. To date, 11 integrin α -chains associated with the $\beta 1$ -chain have been identified and characterized, $\alpha 1$ - $\alpha 10$ and αv (5).

Several integrins bind the sequence RGD in their respective ligands (1). Of those integrins identified so far, $\alpha 4$ -, $\alpha 5$ -, $\alpha 8$ -, $\alpha I I b$ - and αv -chains form heterodimers
30 that mediate RGD-dependent interactions. The ligands containing RGD are generally found in the interstitial type of extracellular matrix. Major non-RGD dependent ligands include various collagen and laminin isoforms. Although both collagens and laminins contain the RGD

sequence in their primary sequences, these RGD sequences are cryptic (6-9) and normally not accessible to cells in the native proteins, but they may be exposed during growth and reorganization events of the extracellular
5 matrix.

Another subdivision of integrins can be made based on structural similarities of the α -chains. A number of integrins contain an extracellular I-domain (10,11) which is homologous to collagen binding A-domains present in
10 von Willebrand factor (12). The I-domain constitutes an inserted domain of approximately 200 amino acids which is present in 8 known integrins ($\alpha 1$, $\alpha 2$, $\alpha 10$, αL , αM , αX , αD and αE) (5,10). Structural analysis of integrin I-domains crystallized in the presence of Mg^{2+} have revealed the
15 presence of a characteristic "MIDAS" (metal ion dependent adhesion site) motif, shown to be critical for ligand binding (13). Integrin α -chains containing the I-domain are not cleaved into heavy and light chains, although the rat $\alpha 1$ chain possesses a proteolytic cleavage site near
20 the membrane spanning region (14,15). For I-domain integrins the principal ligand binding sites are found within the I-domain (10). Known ligands for I-domains found within the $\beta 1$ integrin subfamily include laminins and collagens ($\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins) (16-19), and Echo-
25 virus ($\alpha 2\beta 1$ integrin) (20).

Structure comparisons have suggested that integrins fold into a so-called 7-bladed β -propeller structure which forms one globular domain with the ligand binding region on the upper surface (21). The I-domain is in-
30 serted between blade 2 and 3 in this propeller and divalent cation binding sites are located on the lower surface in blades 4-7 (22,23). Studies of $\beta 2$ integrins have revealed that proper folding of the $\beta 2$ -chain is dependent on the presence of the αL -chain but that the I-
35 domain folds independently of other structural elements in the α - and β -chains (24). In integrin α -chains, a less conserved stalk region separates the predicted β -pro-

pellor from the short transmembrane region. This stalk region is possibly involved in transducing conformational changes between the extracellular and intracellular regions, as well as mediating protein-protein interactions.

5 Although integrins take part in cell signalling events, the cytoplasmic tail is short and lacks enzymatic activity. The sequence GFFKR is conserved in a majority of integrin α -subunits cytoplasmic tails and has been shown to be important for calreticulin binding (25).

10 Cellular interactions with the extracellular matrix during muscle formation and in muscular dystrophy have received increased interest during the past years. In the early 1960's a mutant was described in *Drosophila* which was characterized by the detachment of muscles from
15 their attachment points at the time of the first embryonic muscle contraction, causing the embryos to assume a spheroid shape (26). The mapping of the molecular defect in the lethal myspheroid mutant in 1988 to an integrin β -chain (27), was the first evidence for a role
20 of integrins in maintaining muscle integrity. More recently, refined analysis of *Drosophila* mutants have indicated distinct roles for integrins in muscle endpoint attachments and sarcomere structure (28). The *Drosophila* integrins are all cleaved α -chains and share many fea-
25 tures with vertebrate integrins such as the ability to cluster into focal contacts (29).

The finding that inactivation of the $\alpha 7$ integrin gene in mouse (30), as well as mutations in the human ITGA7 gene (31), both cause muscular dystrophy affecting
30 mainly muscle attachment points, indicates a striking conservation of integrin function during evolution. Of the 11 members of the $\beta 1$ subfamily, $\alpha 7$ exists as a major integrin α -chain (32,33) associated with the $\beta 1 D$ integrin chain in the adult skeletal muscle sarcolemma (34).
35 Intriguingly, mutations in the basement membrane protein laminin $\alpha 2$ -chain (35-37) cause a more severe disease than that observed for the laminin receptor integrin $\alpha 7 \beta 1$

(30). This indicates that other receptors for laminins exist in muscle.

A novel integrin has recently been identified on cultured human fetal muscle cells (38). The present invention is related to, inter alia, the cloning and
5 characterization of this novel I-domain containing, β 1-associated integrin chain, which is expressed in muscle tissues.

SUMMARY OF THE INVENTION

10 The full-length cDNA for this integrin subunit, α 11, has now been isolated. The open reading frame of the cDNA encodes a precursor of 1188 amino acids. The predicted mature protein of 1166 amino acids contains 7 conserved FG-GAP repeats, an I-domain with a MIDAS motif, a short
15 transmembrane region and a unique cytoplasmic domain of 24 amino acids containing the sequence GFFRS. α 11, like other I-domain integrins, lacks a dibasic cleavage site for generation of a heavy and a light chain, and contains three potential divalent cation binding sites in re-
20 peats 5-7. The presence of 22 inserted amino acids in the extracellular stalk portion (amino acids 804-826) distinguishes the α 11 integrin sequence from other integrin α -chains. Amino acid sequence comparisons reveal the highest identity of 42% with α 10 integrin chain. Immuno-
25 precipitation with antibodies to α 11 integrin captures a 145 kD protein, distinctly larger than the 140 kD α 2 integrin chain when analyzed by SDS-PAGE under non-reducing conditions. Fluorescence in situ hybridization maps the integrin α 11 gene to chromosome 15q23, in the
30 vicinity of an identified locus for Bardet-Biedl syndrome. Based on Northern blotting integrin α 11 mRNA levels are high in adult human uterus and in heart, and intermediate in skeletal muscle and some other tissues tested. During in vitro myogenic differentiation, α 11
35 mRNA and protein are up-regulated. Studies of ligand binding properties show that α 11 β 1 binds collagen type

I Sepharose and cultured muscle cells localize $\alpha 1\beta 1$ into focal contacts on collagen type I.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates in its different
5 aspects to the following:

A recombinant or isolated integrin subunit $\alpha 11$ comprising essentially the amino acid sequence shown in SEQ ID No. 1, or homologues or fragments thereof.

The invention also encompasses integrin homologues
10 of said integrin, isolated from other species, such as bovine integrin heterodimer comprising a subunit $\alpha 11$ in association with a subunit β , preferably $\beta 1$, as well as homologues isolated from other types of human cells or from cells originating from other species.

The term "homologues" in the context of the present
15 invention is meant to imply proteins of a common evolutionary origin, having identical or similar functions, specifically requiring evidence based on gene structure and not merely a similarity of protein
20 structure.

The invention also encompasses a process of
producing a recombinant integrin subunit $\alpha 11$ comprising
essentially the amino acid sequence shown in SEQ ID No.
1, or homologues or fragments thereof, which process
25 comprises the steps of

a) isolating a polynucleotide comprising a nucleotide sequence coding for an integrin subunit $\alpha 11$, or homologues or fragments thereof,

b) constructing an expression vector comprising the
30 isolated polynucleotide,

c) transforming a host cell with said expression vector,

d) culturing said transformed host cell in a culture
medium under conditions suitable for expression of integrin subunit $\alpha 11$, or homologues or fragments thereof, in
35 said transformed host cell, and, optionally,

e) isolating the integrin subunit $\alpha 11$, or homologues or fragments thereof, from said transformed host cell or said culture medium. The transformation can be performed *in vitro*, *in situ* or *in vivo*.

5 In further aspects, the invention encompasses:

- A process of providing an integrin subunit $\alpha 11$, or homologues or fragments thereof, whereby said subunit is isolated from a cell in which it is naturally present.

10 - An isolated polynucleotide comprising a nucleotide coding for said integrin subunit $\alpha 11$, or for homologues or fragments thereof, which polynucleotide comprises essentially the nucleotide sequence shown in SEQ ID No. 1 or suitable parts thereof.

15 - An isolated polynucleotide or oligonucleotide which hybridises to a polynucleotide or oligonucleotide encoding said integrin subunit $\alpha 11$ or homologues or fragments thereof, wherein said isolated polynucleotide or oligonucleotide fails to hybridise to a DNA or RNA encoding an integrin subunit $\alpha 10$.

20 - A vector comprising a polynucleotide or oligonucleotide coding for said integrin subunit $\alpha 11$, or homologues or fragments thereof, which polynucleotide or oligonucleotide comprises the nucleotide sequence shown in SEQ ID No. 1 or parts thereof.

25 - A vector comprising a polynucleotide or oligonucleotide which hybridises to a DNA or RNA encoding an integrin subunit $\alpha 11$ or homologues or fragments thereof, wherein said polynucleotide or oligonucleotide fails to hybridise to a DNA or RNA encoding an integrin subunit
30 $\alpha 10$.

- A cell containing the vector as defined above.

- A cell generated during the process as defined above, in which a polynucleotide or oligonucleotide coding for said integrin subunit $\alpha 11$, or homologues or
35 fragments thereof, which polynucleotide or oligonucleotide comprises essentially the nucleotide sequence shown

in SEQ ID No. 1 or parts thereof, has been stably integrated in the cell genome.

- Binding sites of the amino acid sequence of the integrin subunit $\alpha 11$, or of homologues or fragments thereof, said binding sites having the capability of binding specifically to entities chosen from the group comprising proteins, peptides, carbohydrates, lipids, natural integrin binding ligands, polyclonal and monoclonal antibodies, and fragments thereof.

- Binding entities having the capability of binding specifically to integrin subunit $\alpha 11$ comprising the amino acid sequence of SEQ ID No. 1 or to homologues or fragments thereof, preferably chosen from the group comprising proteins, peptides, carbohydrates, lipids, natural integrin binding ligands, polyclonal and monoclonal antibodies, and fragments thereof.

- A recombinant or isolated integrin heterodimer comprising a subunit $\alpha 11$ and a subunit β , in which the subunit $\alpha 11$ comprises essentially the amino acid sequence shown in SEQ ID No. 1, or homologues and fragments thereof. Said subunit β is preferably $\beta 1$.

- A process of producing a recombinant integrin heterodimer comprising a subunit $\alpha 11$ and a subunit β , in which the subunit $\alpha 11$ comprises essentially the amino acid sequence shown in SEQ ID No. 1, or homologues or fragments thereof, which process comprises the steps of

a) isolating one polynucleotide comprising a nucleotide sequence coding for a subunit $\alpha 11$ of an integrin heterodimer and, optionally, another polynucleotide comprising a nucleotide sequence coding for a subunit β of an integrin heterodimer, or polynucleotides or oligonucleotides coding for homologues or fragments thereof having similar biological activity,

b) constructing an expression vector comprising said isolated polynucleotide coding for said subunit $\alpha 11$ optionally in combination with an expression vector com-

prising said isolated nucleotide coding for said subunit β ,

c) transforming a host cell with said expression vector or vectors, which transformation may be performed
5 *in vitro*, *in situ* or *in vivo*,

d) culturing said transformed host cell in a culture medium under conditions suitable for expression of an integrin heterodimer comprising a subunit $\alpha 11$ and a subunit β , or homologues or fragments thereof, in said
10 transformed host cell, and, optionally,

e) isolating the integrin heterodimer comprising a subunit $\alpha 11$ and a subunit β , or homologues or fragments thereof, or the $\alpha 11$ subunit thereof from said transformed host cell or said culture medium.

15 - A process of providing an integrin heterodimer comprising a subunit $\alpha 11$ and a subunit β , or homologues or fragments thereof having similar biological activity, whereby said integrin heterodimer is isolated from a cell in which it is naturally present.

20 - A cell containing

i) a first vector, said first vector comprising a polynucleotide or oligonucleotide coding for a subunit $\alpha 11$ of an integrin heterodimer, or for homologues or parts thereof, which polynucleotide or oligonucleotide
25 comprises essentially the nucleotide sequence shown in SEQ ID No. 1 or parts thereof, and

ii) a second vector, said second vector comprising a polynucleotide or oligonucleotide coding for a subunit β of an integrin heterodimer, or for homologues or fragments thereof.
30

- Binding sites of an integrin heterodimer as defined above, or of homologues or fragments thereof, said binding sites having the capability of binding specifically to entities chosen among the group
35 comprising proteins, peptides, carbohydrates, lipids, natural integrin binding ligands, polyclonal and monoclonal antibodies, and fragments thereof.

- Binding entities having the capability of binding specifically to said integrin heterodimer, or to homologues or fragments thereof, or a subunit $\alpha 11$ thereof. Said subunit β is preferably $\beta 1$. The binding
5 entities are preferably chosen among the group comprising proteins, peptides, carbohydrates, lipids, natural integrin binding ligands, and fragments thereof.

- A fragment of the integrin subunit $\alpha 11$, which fragment is a peptide chosen from the group comprising
10 peptides of the cytoplasmic domain, especially a peptide comprising essentially the amino acid sequence KLGFFRSARRRREPGLDPTPKVLE, of the I-domain, especially a peptide comprising essentially the amino acid sequence from about amino acid No. 159 to about amino acid No. 355
15 of SEQ ID No. 1, and the extracellular extension region, especially a peptide comprising essentially the amino acid sequence from about amino acid No. 804 to about amino acid No. 826 of SEQ ID No. 1.

- A method of producing a fragment of the integrin subunit $\alpha 11$ as defined above, which method comprises a
20 sequential addition of amino acids. This method comprises adding and removing protective groups in a manner known by the man skilled in the art.

- A polynucleotide or oligonucleotide coding for a
25 fragment of the integrin subunit $\alpha 11$ as defined above.

- Binding sites of a fragment as defined above, said binding sites having the capability of binding specifically to entities chosen from the group comprising
30 proteins, peptides, carbohydrates, lipids, natural integrin binding ligands, and fragments thereof.

- Binding entities having the capability of binding specifically to a fragment as defined of the human integrin subunit $\alpha 11$ as defined above. Preferably, said binding entities are chosen from the group comprising
35 proteins, peptides, carbohydrates, lipids, natural integrin binding ligands, and fragments thereof.

- A process of using an integrin subunit $\alpha 11$ comprising essentially the amino acid sequence shown in SEQ ID No. 1 or an integrin heterodimer comprising said subunit $\alpha 11$ and a subunit β , or a homologue or fragment of said integrin or subunit, as a marker or target molecule of cells or tissues expressing said integrin subunit $\alpha 11$, which cells or tissues are of animal including human origin. Especially, said subunit β is $\beta 1$.

In embodiments of this process, said fragment is a peptide chosen from the above defined group.

In one embodiment of said process, the cells are chosen from the group comprising fibroblasts, muscle cells, chondrocytes, osteoblasts, mesenchymally derived cells and stem cells.

Especially, said process is used during pathological conditions involving said subunit $\alpha 11$. Said pathological conditions comprise in one embodiment damage of muscles, muscle dystrophy, fibrosis or wound healing. In another embodiment, said pathological conditions comprise damage of cartilage and/or bone, or cartilage and/or bone diseases. In a still further embodiment, said pathological conditions comprise trauma, rheumatoid arthritis, osteoarthritis or osteoporosis.

In a further embodiment, said process is a process for detecting the formation of cartilage during embryonic development, or for detecting physiological or therapeutic reparation of cartilage and/or muscle, or for selection and analysis, or for sorting, isolating or purification of chondrocytes and/or muscle cells, or for detecting regeneration of cartilage or chondrocytes during transplantation of cartilage or chondrocytes, respectively, or of muscle or muscle cells during transplantation of muscle or muscle cells, respectively, or for studies of differentiation of chondrocytes or muscle cells.

Said process may be and *in vitro*, an *in situ* or an *in vivo* process.

- A process of using binding entities having the capability of binding specifically to binding sites of an integrin subunit $\alpha 11$ as defined above, or of an integrin heterodimer comprising said subunit $\alpha 11$ and a subunit β , or to homologues or fragments thereof, as markers or target molecules of cells or tissues expressing said integrin subunit $\alpha 11$, which cells or tissues are of animal including human origin. Especially, said subunit β is $\beta 1$.

In embodiments of this process, said fragment is as defined above.

In one embodiment, said process is a process for detecting the presence of an integrin subunit $\alpha 11$ comprising the amino acid sequence shown in SEQ ID No. 1, or of an integrin heterodimer comprising said subunit $\alpha 11$ and a subunit β , or of homologues or fragments thereof.

Furthermore, embodiments of this process encompass similar embodiments as defined above in connection with the process of using the integrin subunit $\alpha 11$ as a marker or target molecule.

- A process for detecting the presence of an integrin subunit $\alpha 11$, or of a homologue or fragment of said integrin subunit, as defined above, on cells, whereby a polynucleotide or oligonucleotide chosen from the group comprising essentially a polynucleotide or oligonucleotide as shown in SEQ ID No. 1 is used as a marker under hybridisation conditions, wherein said polynucleotide or oligonucleotide fails to hybridise to a DNA or RNA encoding an integrin subunit $\alpha 10$. Said cells may be chosen from the group comprising muscle cells.

In embodiments of this process, said fragment is as defined above.

Furthermore, embodiments of this process encompass similar embodiments as defined above in connection with the process of using the integrin subunit $\alpha 11$ as a marker or target molecule.

- A pharmaceutical composition comprising as an active ingredient a pharmaceutical agent or an antibody which is capable of using an integrin heterodimer comprising a subunit $\alpha 11$ and a subunit β , or the subunit $\alpha 11$ thereof, or a homologue or fragment of said integrin or subunit $\alpha 11$, as a target molecule.

- A pharmaceutical composition comprising as an active ingredient a pharmaceutical agent or an antibody which is capable of stimulating cell surface expression or activation of an integrin heterodimer comprising a subunit $\alpha 11$ and a subunit β , or the subunit $\alpha 11$ thereof, or homologues or fragments of said integrin or subunit $\alpha 11$. In one embodiment, said composition is for use in stimulating, inhibiting or blocking the formation of muscles, cartilage, bone or blood vessels.

- A vaccine comprising as an active ingredient at least one member of the group comprising an integrin heterodimer, which heterodimer comprises a subunit $\alpha 11$ and a subunit β , or the subunit $\alpha 11$ thereof, and homologues or fragments of said integrin or subunit $\alpha 11$, and a polynucleotide and a oligonucleotide coding for said integrin subunit $\alpha 11$.

- A method of gene therapy, whereby a vector comprising a polynucleotide or oligonucleotide coding for a subunit $\alpha 11$ of an integrin heterodimer, or for homologues or fragments thereof, which polynucleotide or oligonucleotide comprises essentially the nucleotide sequence shown in SEQ ID NO: 1 or parts thereof, and optionally a second vector comprising a polynucleotide or oligonucleotide coding for a subunit β of said integrin heterodimer, is administered to a subject suffering from pathological conditions involving said subunit $\alpha 11$.

- A method of using binding entities having the capability of binding specifically to binding sites of a integrin subunit $\alpha 11$ comprising substantially the amino acid sequence shown in SEQ ID No. 1, or of an integrin heterodimer comprising said subunit $\alpha 11$ and a subunit β ,

or to homologues or fragments thereof, for promoting adhesion of cells.

- A method of using an integrin heterodimer comprising an integrin subunit $\alpha 11$ and a subunit β , or
5 the subunit $\alpha 11$ thereof, or homologues or fragments of said integrin or subunit $\alpha 11$, as a target for anti-adhesive drugs or molecules in tissues where adhesion impairs the function of the tissue.

- A method of in vitro detecting the presence of
10 integrin binding entities, comprising interaction of an integrin heterodimer comprising a subunit $\alpha 11$ and a subunit β , or the subunit $\alpha 11$ thereof, or homologues or fragments of said integrin or subunit, with a sample, thereby causing said integrin, subunit $\alpha 11$, or homologue
15 or fragment thereof, to modulate the binding to its natural ligand or other integrin binding proteins present in said sample.

- A method of in vitro studying consequences of the interaction of a human heterodimer integrin comprising a
20 subunit $\alpha 11$ and a subunit β , or the subunit $\alpha 11$ thereof, or homologues or fragments of said integrin or subunit, with an integrin binding entity and thereby initiate a cellular reaction. In one embodiment of this method, the consequences of said interactions are measured as altera-
25 tions in cellular functions.

- A method of using a polynucleotide or oligonucleotide encoding an integrin subunit $\alpha 11$ or homologues or fragments thereof as a target molecule.

One embodiment of this method comprises hybridising
30 a polynucleotide or oligonucleotide to the DNA or RNA encoding the integrin subunit $\alpha 11$ or homologue or fragment thereof, which polynucleotide or oligonucleotide fails to hybridise to a polynucleotide or oligonucleotide encoding an integrin subunit $\alpha 10$.

35 - A method of using binding entities having the capability of binding specifically to an integrin subunit $\alpha 10$ comprising the amino acid sequence shown in SEQ ID

No. 1 or SEQ ID No. 2, or an integrin heterodimer comprising said subunit $\alpha 10$ and a subunit β , or to homologues or fragments thereof having similar biological activity, for promoting adhesion of chondrocytes and/or osteoblasts to surfaces of implants to stimulate osseointegration.

- A method of using an integrin heterodimer comprising an integrin subunit $\alpha 11$ and a subunit β , or the subunit $\alpha 10$ thereof, or homologues or fragments of said integrin or subunit $\alpha 10$, as a target for anti-adhesive drugs or molecules in tendon, ligament, skeletal muscle or other tissues where adhesion impairs the function of the tissue.

- A method of stimulating, inhibiting or blocking the formation of cartilage or bone, comprising administration to a subject a suitable amount of a pharmaceutical agent or an antibody which is capable of using an integrin heterodimer comprising a subunit $\alpha 11$ and a subunit β , or the subunit $\alpha 11$ thereof, or homologues or fragments of said integrin or subunit $\alpha 11$, as a target molecule.

EXPERIMENTAL PROCEDURES

Cell cultures

The human fetal myoblast/myotube cultures were derived from clone G6 originating from a thigh muscle of a 73-day old aborted fetus ((39); referred to as G6 hereafter). Cultures of G6 and 2.5 years postnatal human satellite cells XXVI, a gift from Dr. Helen Blau (Stanford University, CA), were grown as reported earlier (39). Human rhabdomyosarcoma cell lines RD (ATCC No. CCL-136) and A204 (ATCC No. CRL-7900) were grown in DMEM (Swedish Agricultural University, Uppsala) supplemented with 10% fetal calf serum.

RNA isolation and cDNA synthesis

Total RNA from G6 and XXVI myoblasts, the same cells differentiated for 3 or 7 days, and RD and A204 cell lines, was isolated using the RNeasy Midi kit (Qiagen)

according to the manufacturer's instructions. Poly-A RNA was extracted from total RNA of G6 and XXVI cells using Dynabeads mRNA DIRECT kit (DYNAL A.S., Norway).

PCR based cloning and generation of human α 11 probes

5 First strand cDNA was generated from 1 μ g of G6 mRNA using a reverse transcription PCR-kit (Perkin-Elmer). Advantage cDNA Polymerase Mix (Clontech) was used in PCR amplifications using two different pairs of primers:

10 (1) 5' ACG GGA GAC GTG TAC AAG TG 3' (forward), 5'-AAA GTG CTG AAC CTC CAC CC-3' (reverse) and (2) 5'-CAC CAT CCA CCA GGC TAT GC -3' (forward), 5'-TTA GCG TTC CGT TAT AAA CA -3' (reverse). The PCR conditions were: 94°C, 4 min. ("hot start"); 94°C, 30 s; 55°C, 30 s; and 72°C, 1 min., for 25 cycles. Two products, named PCR1 and PCR2,

15 were obtained (figure 1), subcloned into the plasmid vector TA (Invitrogen), and sequenced. A single product of 1,4 kb in size, named PCR 3 (figure 1), was amplified using primers 1 (forward) and 2 (reverse), and human heart Marathon-Ready cDNA (Clontech) as template. Anneal-

20 ing temperatures in the applied touch-down program were: 68°C, 1 min., 5 cycles; 65°C, 1 min., 5 cycles; 60°C, 1 min, 25 cycles. Other steps were as described above. After the final cycle the reactions were extended for additional 7 min. at 72°C followed by a hold step at 4°C.

25 To obtain a sequence covering the 5' end, Rapid Amplification of cDNA Ends (RACE) was employed according to the manufacturer's instructions (Marathon cDNA Amplification kit, Clontech) using cDNA prepared from G6 mRNA and the gene specific antisense primer: 5'-CTT GGA GAA CCT GAA

30 GTT GGA GTT GAC -3'. Amplification was carried out applying the "touch-down" program (see above). To identify relevant products, 10 μ l of each RACE product was resolved on 1% agarose gel and subjected to Southern blot analysis as described previously (40). PCR2 (see above)

35 was labeled with [α -³²P]dCTP (Amersham Pharmacia Biotech, Sweden) using the RedyPrimeII DNA labeling system (Amersham Pharmacia Biotech, UK), and used as a hybridi-

zation probe. One specific signal was detected. Corresponding cDNA was purified (Gel Extraction kit, Quagen), cloned into the TA vector and sequenced (see figure 1).

Screening of cDNA libraries

5 A λ ZAP custom made G6 cDNA library (Stratagene, USA) was screened with PCR2 (see above) as a probe. The screening procedure (carried out as described in (40)) resulted in two clones representing the 5' non-coding region and the beginning of the coding part of integrin α 11 (figure 1). To obtain an additional sequence, a human uterus 5'-stretch λ gt11 cDNA library (Clontech) was screened with a mixture of PCR1 and PCR2 as probes. The probes were labeled with [α - 32 P]dCTP using the Ready-To-Go DNA labeling beads (Amersham Pharmacia Biotech, Sweden). Three clones 15 (1.1-1.3 in figure 1) representing parts of α 11 cDNA, were obtained. Rescreening of the human uterus 5'-stretch λ gt11 cDNA library with the probe λ 290 (corresponding to 2183-2473 in Fig. 1) yielded three more clones (2.1-2.3, figure 1) covering the rest of α 11 cDNA. Positive clones were 20 plaque purified, the phage DNA isolated using the Lambda Midi kit (Qiagen) and then sub-cloned into the Bluescript SK or pUC19 plasmid vectors before sequencing.

Northern hybridization

25 A filter containing 6 μ g of the poly-A RNA from G6 and XXVI cells and 10 μ g of the total RNA from RD and A204 cell lines, and a Human Multiple Tissue Northern Blot containing poly-A RNA from adult human tissues (Clontech), were hybridized at 68°C in ExpressHyb solution (Clontech) with probes labeled as described above. 30 The probes used were PCR1, PCR2, cDNA clone 1.3 (figure 1), 3RA (1.8 kb cDNA specific for human integrin α 1 mRNA, a generous gift from E.E. Marcantonio (Columbia University, New York), a 1.1 kb cDNA clone recognizing human G3PHD mRNA and a 1.8 kb cDNA clone recognizing human β - 35 actin (both from Clontech).

cDNA sequencing and sequence analysis

All PCR fragments and cDNA clones were sequenced on both strands either manually (29) or using ABI 310 Genetic Analyzer automatic sequencer. Sequences were analyzed with the aid of MacVector™ 6.0, DNA Star, Faktura™NEW 1.2.0, and Sequence Navigator 1.0.1 software programs. A distance tree of all I-domain containing integrin α subunits was assembled using SEAVIEW and PHYLO-WIN softwares (41). Percent similarity between every two members in the I-domain integrin subfamily was calculated by a formula $I=(1-D) \times 100$, where "I" is identity and "D" is distance.

Antibodies

A polyclonal antiserum (α 11 cyt) was produced against the peptide CRREPGLDPTPKVLE from the integrin α 11 cytoplasmic domain. Peptide synthesis and conjugation to Keyhole limpet hemocyanin, immunization of rabbits and affinity purification was performed at Innovagen AB (Lund, Sweden). The monoclonal antibody Mab 13 against integrin β 1 was obtained from S.K. Akiyama (NIEHS, NIH). Monoclonal antibodies to integrin α 1 (clone FB12, sold as MAB 1973) and integrin α 2 (clone BHA2.1 sold as Mab 1998) were both obtained from Chemicon, Temecula, CA. The monoclonal antibody to vinculin (clone hVIN-1) was from Sigma (Saint Louis, MO, USA). Secondary fluorescent antibodies (CY3™-coupled goat-anti rabbit IgG and FITC-coupled goat anti-mouse IgG of multiple labeling grade) were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

Immunoprecipitation and SDS-PAGE

G6 and XXVI cells were labeled with [35 S] cysteine/methionine and subjected to immunoprecipitation and SDS-PAGE as reported previously (38). The two-step procedure used to dissociate integrin heterodimers was carried out as follows. After incubation of samples with β 1 antibody and capture with GammaBind G Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden), 100 μ l of 1% SDS was

added to the washed beads which were then boiled for 5 minutes. 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl and 1% Triton X-100 was added to a final volume of 1ml and the lysate was incubated with GammaBind G Sepharose for 1
5 hour. The incubation with GammaBind G was performed in order to ensure that no reactive $\beta 1$ antibodies remained. After removal of GammaBind G Sepharose, $\alpha 11$ integrin antibody was added for additional 2 hours, followed by capture with protein A Sepharose (Amersham Pharmacia
10 Biotech) and boiling in SDS-PAGE sample buffer.

Chromosomal localization

Chromosomal localization of the human integrin $\alpha 11$ was performed by using a combination of FISH (Fluorescent In Situ Hybridization) technique and DAPI
15 (4',6-diamidino-2-phenylindole) banding essentially as described earlier (42). As a hybridization probe, the 1.4 kb RT-PCR product PCR3 was used.

Surface iodination and affinity chromatography

Cultured XXVI cells were surface iodinated as
20 described (38). Labeled cells were solubilized in 1 ml of solubilization buffer (10 mM Tris-HCl pH 7.4, 15 mM NaCl, 1% Triton X-100, 1mM $MgCl_2$, 1 mM $CaCl_2$, 1mM $MnCl_2$), centrifuged at 14000 g for 20 min., and soluble membrane proteins were applied to a collagen type I Sepharose
25 (bovine collagen type I from Vitrogen (Collagen Corp., Palo Alto) coupled to CNBr-activated Sepharose CL-4B at 3 mg/ml gel as described (14)), equilibrated in solubilization buffer. Following a one hour incubation the column was washed extensively with buffer A (10 mM
30 Tris-HCl pH 7.4, 50 mM NaCl, 1 mM $MnCl_2$, 0.1% Triton X-100) and by 10 column volumes of buffer A without NaCl. Bound proteins were eluted with 20 mM EDTA, 10 mM Tris-HCl pH 7.4, 0.1% Triton X-100. Peak fractions were pooled and concentrated by immunoprecipitation with $\beta 1$ integrin
35 and $\alpha 11$ integrin antibodies as described under Immuno-precipitation and SDS-PAGE. Eluted frations and captured

proteins were analyzed on 7.5% SDS-PAGE gels followed by autoradiography.

Indirect immunofluorescence

Cells cultured on coverslips were washed in serum-free medium and fixed for 8 min. in acetone at -20°C . Non-specific binding sites were blocked by incubating with 10% goat serum diluted in phosphate buffered saline. In the double immunofluorescence staining protocol, primary antibodies (anti- α 11 cyt (rabbit antibody) and anti-vinculin (mouse antibody)) were simultaneously incubated with fixed cells for 1.5 hours at $+37^{\circ}\text{C}$. Specifically bound antibodies were detected using anti-rabbit Cy3 IgG and anti-mouse FITC IgG. Stained cells were mounted in Vectashield™ mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA) and visualized and photographed under a Zeiss light microscope equipped with optics for observing fluorescence.

RESULTS AND DISCUSSION

20 cdna cloning of a novel integrin α -chain

In order to determine the nature of the integrin chain that we had previously characterized on human fetal muscle cells and named α mt (38), a number of approaches were used. Applying PCR with mRNA from fetal muscle cells as template together with degenerate primers to conserved regions of integrin α subunits (43) we amplified cDNA for α 1, α 4, α 5, α 6 and α v integrin chains (data not shown), but failed to amplify the novel integrin. However, while searching through the literature we came across two integrin sequences obtained in a subtractive hybridization protocol comparing human primary myoblasts with the rhabdomyosarcoma cell line RD (44). After having confirmed that these sequences could be amplified by PCR from human fetal G6 myoblast cDNA, PCR was performed assuming that these sequences were derived from the same transcript. In this manner a 1.4 kb cDNA fragment with integrin-like sequence was obtained. Screening of a human fetal myo-

blast cDNA library and 5' RACE yielded additional 5' sequence. We determined the mRNA expression pattern in a number of human tissues (see below) and observed a high mRNA expression in the uterus. Screening of a uterus cDNA library resulted in the identification of the complete open reading frame. A schematic illustration of the cloning strategy is shown in figure 1.

cDNA sequence and predicted amino acid sequence of $\alpha 1$ integrin chain

By sequence analysis of cDNA clones and 5' RACE products we obtained a continuous sequence of 3983 nucleotides (nt) composed of 90 nt 5' non-coding sequence, 3564 nt open reading frame, and 326 nt 3' non-coding sequence. Translation of the sequence predicts an integrin α -chain like precursor of 1188 amino acids including a 22 amino acid long signal peptide (fig. 2, GenBank accession No. AF137378). The mature 1166 amino acid long peptide is larger than any other currently identified integrin α -chain (the closest being αE , composed of 1160 amino acids (45). The 1115 amino acid long predicted extracellular domain contains 7 FG-GAP repeats in the amino-terminal end with an inserted I-domain between repeats 2 and 3. The I-domain consists of 195 amino acids and includes a conserved MIDAS motif. In addition to the metal chelating site in the I-domain, three additional potential divalent cation binding motifs with the consensus sequence DXD/NXDXXXD are present in repeats 5-7. A total of 20 cysteines are located in the extracellular domain. Of these, 16 are conserved in the most closely related integrin $\alpha 10$ and $\alpha 1$ chains and they may contribute to intramolecular disulphide bonds. The two non-conserved cysteines found at positions Cys 606 and Cys 988 most likely represent free unpaired cysteines while the two non-conserved cysteines Cys 806 and Cys 817 may pair to form a disulphide bond. Mapping of the cysteines in the suggested β -propeller structure shows that the first three disulphide bonds are likely to stabilize

blades one and two of the β -propeller whereas the remaining bonds are found outside the propeller region, in the stalk region towards the transmembrane domain. 16 potential N-glycosylation sites are present in $\alpha 11$. A
5 search for sequence motifs reveals the presence of a 22 amino acid leucine zipper motif starting at position 951, and a 17 amino acid sequence starting at position 1082, which is similar to sequences found in G-protein coupled receptors. These sequences might represent functional
10 domains of importance for protein-protein interactions.

The transmembrane region (amino acids 1142-1164) is 23 amino acids long and is followed by a cytoplasmic tail of 24 amino acids. The cytoplasmic tail contains the sequence GFFRS instead of the conserved GFFKR sequence,
15 found in all other α -chains except $\alpha 8$ - $\alpha 10$. It will be interesting to determine the importance of this sequence in defining the cytoplasmic domain as well as its possible ability to bind calreticulin and other intracellular components.

20 Comparison of integrin $\alpha 11$ chain with other integrin α chains

Alignment of the predicted $\alpha 11$ integrin amino acid sequence with other integrin sequences shows the highest overall identity with $\alpha 10$ (42% identity), $\alpha 1$ (37% identity), and $\alpha 2$ (35% identity), followed by the remaining
25 I-domain containing integrin subunits. Of the non I-domain containing integrins, $\alpha 4$ and $\alpha 9$ are the most similar to $\alpha 11$. A distance tree shows that $\alpha 10$ and $\alpha 11$ form a separate branch from the most closely related $\alpha 1$ and $\alpha 2$ integrin chains (fig. 3). The similarity with
30 other integrins is particularly high in the N-terminal β -propeller part but lower in the stalk region. Comparison of $\alpha 1$ integrin with $\alpha 2$ integrin has pointed to the presence of a 38-residue insert in the β -propeller region
35 of $\alpha 1$ integrin chain (15). Like $\alpha 1$ chain, $\alpha 11$ also contains inserted amino acids not present in the other I-domain containing integrin chains. however, in the $\alpha 11$

chain these are found within the stalk region at amino acids 804-826. The exact border of the predicted insertion varies depending on the alignment method and the parameters chosen, but is predicted to span at least 22 amino acids. The insert shows no significant similarity to other integrin sequences and contains two cysteines likely to form a disulphide bond (see fig. 2). We do not believe that the predicted inserted sequence represents a cloning artifact since it is present in three independently analysed clones. Other examples of non I-domain inserted sequences are found in the *Drosophila* α PS2 chain, where developmentally regulated splicing in the ligand binding region modulates ligand affinity (46). In $\alpha 7$ integrin chain, splicing in the extracellular domain between predicted blades 2 and 3 in the β -propeller generates X1 and X2 variants, affecting the binding to laminin-1 in a cell-specific manner (47). In the more closely related $\alpha 1$ integrin chain the 38 extra amino acids are present in a position that is predicted to be in the beginning of the sixth blade of the 7-bladed propeller. So far there is no evidence that the extra amino acids in either $\alpha 1$ or $\alpha 11$ arise by alternative splicing. In $\alpha 11$ the predicted inserted region is outside the β -propeller and most likely does not directly affect ligand binding, but might instead be involved in modifying protein-protein interactions or be important for outside-in or inside-out signalling. In this regard it is interesting to note that tetraspan proteins by binding to the stalk region of certain integrin α -chains can recruit PI-4 kinase and protein kinase C to integrin complexes (48). Likewise the extracellular membrane-proximal parts of certain integrin α -chains have been shown to be involved in Shc-mediated integrin signalling (49).

Analysis of sequences identified during screening for genes upregulated during tadpole regression revealed a partial sequence, which at the time was reported to show the highest similarity to integrin $\alpha 1$ (41% identity)

(50). This sequence, when translated (amino acids 1-116), shows 71% identity to human $\alpha 11$ and thus most likely represents the *Xenopus* orthologue of $\alpha 11$ rather than that of the $\alpha 1$. These data suggest that $\alpha 11$ is well conserved during evolution.

Chromosomal localization of the integrin $\alpha 11$ gene

A fluorescent cDNA probe was used for in situ hybridization on metaphase chromosome spreads. The analysis shows that the integrin $\alpha 11$ gene (ITGA11) is located on chromosome 15q23 (fig. 4). The genes for I-domain containing integrins $\alpha 1$ and $\alpha 2$ are both present on chromosome 5 (51,52), just as the genes for the closely related $\beta 2$ integrin associated α -chains all map to chromosome 16 (53). Interestingly, the $\alpha 11$ gene and the closely related $\alpha 1$ and $\alpha 2$ genes, map to different chromosomes. It will be of evolutionary interest to determine the chromosomal localization of the integrin $\alpha 10$ gene. Curiously, a form of Bardet-Biedl syndrome characterized by retinitis pigmentosa, polydactyly, obesity, hypogenitalism, mental retardation, and renal anomalies maps to 15q22-23 (54). Future studies will clarify a possible linkage of ITGA11 to Bardet-Biedl syndrome.

Expression pattern of $\alpha 11$ mRNA in adult tissues

Northern blot analysis of mRNA from various adult human tissues shows the highest level of expression of $\alpha 11$ in adult human uterus. A strong signal is also noted in heart, while intermediate levels of $\alpha 11$ mRNA are present in skeletal muscle and intermediate to low levels in other adult tissues tested (fig. 5 and data not shown). For a comparison, the same blot was probed for the closely related $\alpha 1$ integrin mRNA (fig. 5). A striking difference in the expression levels of $\alpha 1$ and $\alpha 11$ was observed in the smooth muscle rich uterus, which appears to lack $\alpha 1$. Immunohistochemical analysis and in situ hybridizations will elucidate the detailed distribution of $\alpha 11$ protein and mRNA in muscle and other tissues. Neither $\alpha 1$ (33) nor $\alpha 2$ (55) are present in muscle fibers, and the

distribution of $\alpha 10$ in skeletal muscle tissues is not known (5). Hence, no I-domain containing integrin has so far been reported to be expressed in the skeletal muscle sarcolemma. Recently the gene for $\alpha 1$ integrin was inactivated in mice, resulting in mice with an apparently normal phenotype (56). More careful analysis revealed a phenotype characterized by a hypocellular skin (57) and aberrant regulation of collagen synthesis (58). It will be interesting to compare sites of overlapping expression between $\alpha 1$, $\alpha 2$ and $\alpha 10$ integrins, and use reagents to $\alpha 10$ and $\alpha 11$ to examine possible functional compensatory mechanisms in $\alpha 1$ integrin-deficient mice.

Biochemical characterization of $\alpha 11$ protein

Following the cloning of the full-length $\alpha 11$ integrin cDNA it was essential to determine if the predicted amino acid sequence was identical to the novel uncleaved $\beta 1$ integrin-associated α -chain that we had previously noted to be upregulated during in vitro differentiation of human myoblasts (38). To answer this question we raised antibodies to the cytoplasmic tail of the integrin $\alpha 11$ chain. Immunoprecipitation from the human satellite cells showed that the antibodies precipitated a 145 kDa $\alpha 11$ band associated with a 115 kDa $\beta 1$ band (fig. 6, panel A) in SDS-PAGE under non-reducing conditions. Under reducing conditions the $\alpha 11$ band migrated as 155 kDa (see fig. 6, panel B). From the translated amino acid sequence an Mr of 133 400 is predicted for the $\alpha 11$ chain. Taking the 16 potential glycosylation sites into account this fits well with the observed 155 kDa band in SDS-PAGE. Under non-reducing conditions the 145 kDa band is distinctly larger than $\alpha 2$ (fig. 6, panel A) and $\alpha 10$ integrin chains which co-migrate as 140 kDa bands and $\alpha 11$ migrates well below the 180 kDa integrin $\alpha 1$ band. The $\alpha 2$ (59) and $\alpha 10$ (5) chains both contain 10 potential glycosylation sites whereas $\alpha 1$ contains 26 glycosylation sites (60). The intermediate size of $\alpha 11$ in SDS-PAGE compared with $\alpha 1$

and $\alpha 2/\alpha 10$ is thus most likely a result of differential glycosylation.

To show that $\alpha 11$ is associated with the $\beta 1$ subunit a two-step immunoprecipitation procedure was performed.

5 Integrins were first precipitated with a monoclonal anti- $\beta 1$ integrin antibody and GammaBind G captured integrins were then dissociated by boiling in 1% SDS. In the second step, SDS was diluted tenfold and antibodies to $\alpha 11$ were added. As shown in fig. 6 panel A antibodies to $\alpha 11$ immu-
10 noprecipitate only the 145 kDa band from the dissociated precipitate initially captured with $\beta 1$ antibodies.

Induction of $\alpha 11$ mRNA and protein during myogenic differentiation in vitro

It has previously been determined that αmt is the
15 major integrin α -chain that is up-regulated during myogenic differentiation on human fetal myoblasts in vitro (38). To compare $\alpha 11$ levels in myoblasts and myotubes, immuno-precipitates were analyzed from myoblast cultures in pro-liferation medium, and from parallel
20 cultures allowed to differentiate and form myotubes in differentiation medium for 7 days. Immunoprecipitation with both $\beta 1$ and $\alpha 11$ antibodies showed that $\alpha 11$, like αmt , is strongly up-regulated at the protein level in differentiation cultures of human fetal muscle cells and
25 satellite cells (fig. 6, panel B). To determine if the up-regulation occurs at the mRNA or protein level we analyzed $\alpha 11$ mRNA from different differentiation stages (day 1, day 3 and day 7) (fig. 6, panel C). Already at day 3 in differentiation medium a strong up-regulation of
30 $\alpha 11$ mRNA was noted, establishing that the up-regulation of $\alpha 11$ integrin protein occurs as a result of increased transcription or mRNA stability. Based on similar SDS-PAGE migration patterns, similar behavior under reducing conditions, association with $\beta 1$ integrin chain, and up-
35 regulation during in vitro differentiation of human fetal myoblasts, the present data show that $\alpha 11$ integrin is identical with αmt .

Analysis of mRNA from the two rhabdomyosarcoma cell lines RD and A204 (fig. 6, panel C) did not provide evidence for the presence of $\alpha 11$ in either cell line. Based on the observed up-regulation of $\alpha 11\beta 1$ in human fetal muscle cells and the presence of $\alpha 11$ message in adult muscle we suggest that $\alpha 11$ integrin might be involved in early steps of muscle formation and that it in adult muscle tissues may fulfill a stabilizing role. The $\alpha 7$ integrin subunit is a major $\beta 1$ -associated integrin chain in muscle, but genetic deletion of $\alpha 7$ leads to a fairly mild muscular dystrophy (30).

Ligand binding specificity of $\alpha 11\beta 1$ integrin

So far identified I-domain containing integrins of the $\beta 1$ integrin subfamily all bind collagens (5,15,59). For $\alpha 1$ and $\alpha 2$ this binding capacity has been shown to reside within the I-domain (17,18). To determine if $\alpha 11\beta 1$ also binds collagen we performed collagen type I Sepharose chromatography of membrane proteins from surface-iodinated XXVI satellite cells. Direct analysis of the EDTA eluate revealed weak bands corresponding to the positions of $\alpha 1$, $\alpha 2$, $\alpha 11$ and $\beta 1$ in parallel immunoprecipitations (figure 7, panel 1). The EDTA eluate was concentrated by immunoprecipitation with $\beta 1$ and $\alpha 11$ antibodies. As shown in figure 7, a prominent $\alpha 11$ band is present in the collagen I Sepharose eluate. The relatively weak $\beta 1$ band in the proteins captured with $\alpha 11$ antibodies indicates that the $\alpha 11\beta 1$ heterodimer partly dissociates in the presence of EDTA. To visualize the interaction of $\alpha 11\beta 1$ integrin with collagen I in intact cells, myogenic cells expressing $\alpha 11\beta 1$ were trypsinized and plated on collagen and fibronectin for 1 hour. The ability to form focal contacts was investigated by double immunofluorescence staining for $\alpha 11$ -chain and vinculin. As seen in panel 2 of figure 7 $\alpha 11$ localizes to vinculin positive focal contacts on collagen but not on fibronectin. Binding studies with $\alpha 11$ I-domain expressed as a bacterial GST-fusion protein also confirmed a specific

affinity for collagen I (unpublished M. Höök, R. Rich, R. Owens). Stable transfections of $\alpha 11$ cDNA into cells with various integrin backgrounds will allow a more detailed study of $\alpha 11\beta 1$ interactions with different collagen, and possibly also laminin, isoforms. Combined with in vivo distribution studies of $\alpha 11\beta 1$ this is likely to yield valuable information regarding the in vivo ligands for $\alpha 11\beta 1$ in different tissues.

$\alpha 11$ integrin protein distribution in human embryo

Morphologically normal human embryos (aged from 4 to 8 post-ovulatory weeks) were obtained from legal abortions induced by Mifepristone (RU486) at Hopital Broussais in Paris. All procedures were approved by the Ethical Committee of Saint-Vincent de Paul Hospital in Paris.

Each sample was first examined macroscopically during dissection under a stereo-microscope. The development stage of the embryos was determined using established criteria. Tissues were collected shortly after delivery and frozen within the first 24 h post mortem on dry ice and stored at -80°C until used. Seven micron-thick cryostat sections were mounted on slides previously coated with a 2% 3-aminopropyl-triethoxysilane solution in acetone. The cryosection was left unfixed prior to blocking of non-specific binding sites with 10% goat serum diluted in phosphate buffered saline. For immunofluorescence, the section was incubated with $\alpha 11$ antibodies 1.5 h at $+37^{\circ}\text{C}$. Specifically bound antibodies were detected using goat anti-rabbit Cy3 IgG (Jackson Immunoresearch). The stained tissue section was mounted in VectashieldTM mounting medium (Vector Laboratories Inc.) and visualized and photographed under a Zeiss light microscope equipped with optics for observing fluorescence.

The results obtained are shown in figure 8. High levels of $\alpha 11$ protein were noted around vertebrae (arrows), in intervertebrae disc (asterisks), around ribs

(thin arrows) and around forming cartilage in the forelimb (arrowhead).

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FIGURE LEGENDS

Figure 1. Schematic organization of PCR fragments and cDNA clones representing different parts of the full length sequence of integrin $\alpha 11$ subunit

A. Clones 1.1-1.3 and 2.1-2.3 are from the first and the second round of screening, respectively. Fragment 0.0 represents a 5' RACE product as well as a clone obtained from screening of the G6 library. PCR fragments 1-3 and a SacI fragment of a clone 1.3, $\lambda 290$, are marked with thick lines. Names and positions of all the clones on a scheme are shown in tabulated form in B.

B. Names of the PCR-amplified fragments and cDNA clones shown in A are in the left column, and their positions in the full length cDNA of integrin $\alpha 11$ in the right column.

Figure 2. Nucleotide and deduced amino acid sequence of the human integrin $\alpha 11$ chain

The putative signal peptide is underlined in bold, I-domain is boxed, potential N-linked glycosylation sites are marked with asterisks, cysteines are underlined, potential divalent cation binding motifs are double underlined and the transmembrane domain is underlined with dashes. A 22 amino acid insert is boxed in bold.

Figure 3. A distance tree of the I-domain containing α -integrin subfamily members

A tree was assembled based using ClustalW multiple alignment - based SEAVIEW and PHYLOWIN softwares. A scale at the bottom shows percent identity.

Figure 4. Chromosome mapping of ITGA11 gene by fluorescent in situ hybridization (FISH)

A. Left panel shows the FISH signals on human chromosome 15; right panel shows the same mitotic figure stained with 4',6-diamino-2-phenylindole to identify human chromosome 15.

B. Diagram of FISH mapping result for the probe PCR3 based on a detailed analyses of 10 different images. Each

dot represents the double FISH signals detected on human chromosome 15.

Figure 5. Expression of integrin $\alpha 11$ and $\alpha 1$ subunit mRNAs in adult human tissues

- 5 Integrin $\alpha 11$ mRNA and integrin $\alpha 1$ mRNA were analyzed on a membrane with RNA from various adult human tissues where mRNA loading was normalized with respect to β -actin. Probes used for hybridizations are marked on the left. Size of molecular weight standard is marked to the right.
- 10 Note that the β -actin probe reacts with 2 kb β/γ actin transcripts and the muscle specific 1.8 kb α -actin message.

15 Figure 6. Biochemical characterization of integrin $\alpha 11$ chain and upregulation of corresponding protein and mRNA in myogenic cells

- A. $\alpha 11$ associates with $\beta 1$ integrin chain. Human XXVI and G6 muscle cells were metabolically labeled with [^{35}S] cysteine/methionine and integrins were immunoprecipitated with the indicated antibodies ($\beta 1$, $\alpha 2$ and $\alpha 11$). Evidence
- 20 for the association of integrin $\alpha 11$ with the $\beta 1$ subunit obtained by treating proteins precipitated with anti- $\beta 1$ antibodies with SDS followed by a second precipitation with $\alpha 11$ antibodies (ant- $\alpha 11$ +SDS). Precipitated proteins were resolved on 7.5% SDS-PAGE gels in the absence of
- 25 reducing agents, followed by fluorography.

B. Induction of integrin $\alpha 11$ upon myogenic differentiation in vitro.

- G6 muscle cells were metabolically labeled with [^{35}S] cysteine/methionine when growing in proliferation medium
- 30 (mb-proliferating myoblasts) and after 7 days in differentiation medium) (mt-myotubes). Integrins were precipitated with antibodies to $\beta 1$ and $\alpha 11$ and the precipitates were resolved on 7.5% SDS-PAGE gels both under non-reducing (UNREDUCED) and reducing (REDUCED) conditions.
- 35 Lanes 1, 3, 5 and 7 are immunoprecipitations with the antibody to integrin $\beta 1$, and lanes 2, 4, 6 and 8 with the antibody to integrin $\alpha 11$.

C. Upregulation of integrin $\alpha 11$ mRNA in differentiated myogenic cells.

mRNA was extracted from G6 and XXVI cells growing under proliferating (p) or differentiating (d) conditions for 3 days (d3) or 7 days (d7). Total RNA was isolated from RD and A204 cells. Following separation of RNA on agarose gel and transfer to the membrane, the filter was hybridized with probes to $\alpha 11$ integrin ($\alpha 11$) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Size of bands in RNA standard (in kb) are marked to the right.

Figure 7. Ligand binding properties of $\alpha 11\beta 1$ integrin panel 1: Collagen binding integrins on XXVI cells.

XXVI cells were surface iodinated and integrins were analyzed by immunoprecipitation and collagen I Sepharose affinity chromatography. Immunoprecipitation reveals the presence of $\beta 1$ integrins (lane 1), $\alpha 1\beta 1$ (lane 2), $\alpha 11\beta 1$ (lane 3) and $\alpha 2\beta 1$ (lane 4) at the surface of XXVI cells. EDTA eluted proteins bound to collagen I Sepharose contain weak band in the position of $\alpha 1$, $\alpha 11$, $\alpha 2$ and $\beta 1$ integrin chains (lane 5). Immunoprecipitations with $\beta 1$ integrin antibodies (lane 6) and $\alpha 11$ integrin antibodies (lane 7) confirm the presence of $\alpha 11$ and $\beta 1$ in the EDTA eluate.

panel 2: $\alpha 11\beta 1$ localizes to focal contacts on collagen.

Indirect immunofluorescent visualization of vinculin (A, B) and $\alpha 11$ integrin chain (C, D) in human XXVI satellite cells seeded on collagen type I (A and C) and fibronectin (B and D). Note the localization of integrin $\alpha 11$ chain to focal contacts of cells allowed to attach to collagen and its complete absence on cells seeded on fibronectin. Vinculin is found in focal contacts on both substrates. A and C show the same cell double stained for both antigens. Scale bar is $20\mu\text{m}$.

Figur 8. α 11 integrin protein distribution at 8 weeks of gestation.

- Composite of immunohistochemical staining of sagittal section of human embryo at 8 weeks of gestation. Note
- 5 high levels of α 11 protein around vetrebrae (arrows), in intervertebral disc (asterisks), around ribs (thin arrows) and around forming cartilage in the forelimb (arrowhead).